

USER MANUAL

PIXELGEN SINGLE CELL SPATIAL PROTEOMICS KIT

IMMUNOLOGY PANEL I, Human

PXGIMM001



PIXELGEN
TECHNOLOGIES

Version 1.01

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List of Abbreviations

- AOC Antibody Oligonucleotide Conjugate
- CMR Substance Carcinogenic, Mutagenic, or toxic to Reproduction
- DMSO Dimethyl Sulfoxide
- FBS Fetal Bovine Serum
- MPX Molecular Pixelation
- NGS Next Generation Sequencing
- PBS Phosphate-Buffered Saline
- PCR Polymerase Chain Reaction
- PFA Paraformaldehyde
- PBMC Peripheral Blood Mononuclear Cell
- RT Room Temperature
- SPRI Solid Phase Reversible Immobilization

About this User Manual

This User Manual describes the experimental procedure using the Pixelgen Single Cell Spatial Proteomics Kit, Immunology Panel I, Human (# PXGIMM001) in detail.

Technical Support

For technical support, please contact Pixelgen Technologies at support@pixelgen.com.

1. Product Description

The Pixelgen Single Cell Spatial Proteomics Kit, Immunology Panel I, Human reagent kit can be used to study surface protein expression and spatial arrangement on paraformaldehyde fixed human immune cells in suspension.

The technology behind the SCSP panel is called Molecular Pixelation (MPX). The core steps of the technology are illustrated below.

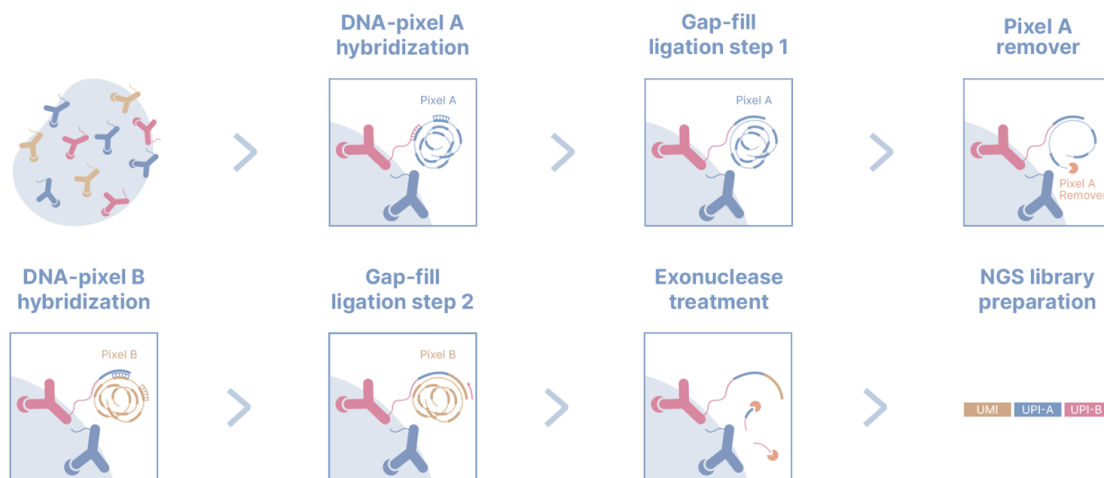


Figure 1. Overview of Molecular pixelation workflow and core steps.

Reagents supplied with the Molecular Pixelation kit

Reagents included in the kit are listed below in Table 1. Each kit contains reagents sufficient for processing of 8 reactions. The reagents are supplied in three individual boxes. Storage temperature and expiration date for components are stated on the label of each box.

Note All reagents for the Pixelgen Single Cell Spatial Proteomics Kit, Immunology Panel I, Human are lot specific and reagents from different kit lots should not be combined.

Product number: PXGIMM001

Table 1. Reagents supplied in the individual kit boxes and their storage temperatures. Bullet point colors correspond to the reagent lid color.

Box 1 - Panel & Pixels PXGIMM001PP store at 4°C	Box 2 - Enzymes PXGIMM001E store at -20°C	Box 3 - Index Primers PXGIMM001IP store at -20°C
<ul style="list-style-type: none"> ● AOC Panel - PP010 ● 2nd antibody - PP028 ● Wash Buffer - PP015 	<ul style="list-style-type: none"> ● Blocking buffer - PP009 ● Pixel A - PP018A ● Pixel A Diluent - BD019A ● Pixel B - PP018B ● Pixel B Diluent - BD019B ● Gap-fill Buffer - BD018 ● Gap-fill Ligase - EE019 ● Gap-fill Polymerase - EE020 ● Pixel A Remover - EE021 ● Exonuclease - EE022 	<ul style="list-style-type: none"> ● PCR Master Mix - MM024 ● Primer 1 - IP023_1 ● Primer 2 - IP023_2 ● Primer 3 - IP023_3 ● Primer 4 - IP023_4 ● Primer 5 - IP023_5 ● Primer 6 - IP023_6 ● Primer 7 - IP023_7 ● Primer 8 - IP023_8

Additional Requirements

Below is a list of equipment, reagents and consumables required to perform the MPX assay. The suggested suppliers and part numbers noted are equivalent to equipment used during optimization and validation of the MPX assay panel.

Third party Instrumentation needed

Table 2a. List of suggested third party equipment needed to perform the analysis workflow.

Equipment			
Description	Product name	Suggested Supplier	Part number
Centrifuge with rotor for 2 mL micro tubes (17 000 x <i>g</i>)	Centrifuge 5418 R - microcentrifuge	Eppendorf®	5418 R
Centrifuge PCR tubes (1000 x <i>g</i>)	Mega Star 4.0R	VWR®	521-2664
Thermocycler/ PCR system	ProFlex™ 3x 32-well PCR System	ThermoFisher®	4484073
Pipettes: <ul style="list-style-type: none"> • 0.5 - 2.5 µl • 2 - 20 µl • 20 - 200 µl • 100 - 1000 µl 	Research® plus	Eppendorf®	J70399L O89082L N23237L N44241L
Hemocytometer	Counting Chambers Bürker-Türk	Karl Hecht Assistent®	40445
or			
Single-use hemocytometer	2-Chip Disposable Hemocytometer	Bulldog Bio®	NHC-D002
Magnet for PCR tubes	DynaMag™-96 Side Magnet	ThermoFisher®	12331D
Light microscope	Microscope trinocular inverted	Sagitta®	63335
PCR product size and quantification instrument	BioAnalyzer™ with a High Sensitivity DNA kit	Agilent®	G2939A

Table 2b. List of suggested third party consumables needed to perform the workflow.

Consumables			
Description	Product name	Suggested Supplier	Part number
0.2mL PCR tubes	FastGene® PCR Tubes 0.2mL	Nippon Genetics Europe	FG-021
1.5mL low adhesion tube	Eppendorf Tubes® low adhesion	BIOplastics™	B74030
Pipette tips: 10 µl 200 µl 1000 µl	OMNITIP™ Sterile, filter tips	ULPlast Sp.z.o.o.	83240 81240 85240

Table 2c. List of suggested third party reagents needed to perform the workflow.

Reagents			
Description	Product name	Suggested Supplier	Part number
1xPBS	PBS, pH 7.4	ThermoFisher®	10010-023
Paraformaldehyde, methanol-free*	Paraformaldehyde 16% Aqueous Sol.	Electron Microscopy Sciences	15710
AMPure beads	AMPure XP reagent, 60mL	Beckman-Coulter® Life science	A63881
TE buffer	Tris-EDTA buffer solution, BioUltra, for molecular biology, pH 8.0	Sigma-Aldrich®	93283

* It is important to use methanol-free paraformaldehyde as methanol can permeabilize the cell membrane and promote protein denaturation.

2. General guidelines & information

Cell input requirements

The protocol consists of several steps where a certain range of cell input is required (Table 3.). Due to cell losses during fixation and overall protocol washing steps, it is important to stay within the range to ensure that enough cells remain.

Validation of cell inputs to fixation step have been confirmed for up to 1 million cells per reaction, AOC binding step has been validated for cell inputs up to 500 000 cells and the MPX step has been validated for up to 100 000 cells per reaction. The cell input range to PCR needs to be within 300 to 1000 cells to ensure software compatibility.

Table 3. Cell inputs and counting requirements for different steps of the protocol.

Step	Cell input range per reaction	Recommended cell input per reaction	Counting, accuracy
Fixation	500 000 - 1 000 000 cells	500 000 - 1 000 000 cells	Required, estimation
AOC binding	50 000 - 500 000 cells	200 000 cells	Optional, estimation
MPX	10 000 - 100 000 cells	20 000 cells	Required, precise
Exonuclease and PCR	300 - 1 000 cells	300 - 1 000 cells, dependent on experimental set-up*	Required, precise

* The number of cells input to PCR will directly impact the read requirements.

Cell counting considerations

Counting of cells after fixation and AOC binding, prior to Molecular Pixelation can be performed using either a hemocytometer or using automated cell counters as the cell numbers are higher during this stage of the workflow.

However, it is important to accurately count and dilute cells prior to Exonuclease & PCR step, as these products will be taken forward to sequencing, and inaccurate cell numbers may result in poor data quality due to insufficient sequencing read amounts. Due to the relatively low cell numbers at this stage, we have found automatic cell counters such as the Countess Automated cell counter (ThermoFisher) to **not** be reliable and could require a significant portion of the reaction to be used for counting.

We therefore highly recommend using manual counting with a hemocytometer or single-use counting chamber when counting cells prior to the Exonuclease step of the Molecular Pixelation workflow. Follow counting guidelines associated with the hemocytometer or single-use counting chamber type used. To ensure accurate counting, duplicate counts for each reaction is highly recommended.

Sample indexing during PCR

The reagent kit is supplied with 8 PCR primer sets, each primer set containing a unique sample index barcode combination, allowing for pooling of samples for sequencing on the same Illumina flow cell (Table 4). It is critical that each individual sample sequenced together in the same run has a unique sample index combination.

Table 4. List of sample indexes supplied with the reagent kit.

index name	i7 index	i5 index* (forward)	i5 index** (reverse complement)
i1	CAATGTGG	CCAACACT	AGTGTGG
i2	AGTTGGCT	CAGTCACA	TGTGACTG
i3	AACGTGAT	TAGTCTCG	CGAGACTA
i4	AACCGAGA	AACGCACA	TGTGCGTT
i5	AGAGTCAA	CAGGTAAG	CTTACCTG
i6	CACTTCGA	ACCATAGG	CCTATGGT
i7	TTTGGGTG	ACACCTCA	TGAGGTGT
i8	ATGTGAAG	CAACACAG	CTGTGTTG

* Forward i5 index should be used for NextSeq® 1000, 2000 (using **v2** Sample Sheet), NovaSeq® 6000 (**v1.0** reagent kits), HiSeq® 2000, 2500, MiSeq®, MiniSeq® (rapid reagents kits)

** Reverse complement i5 index should be used for NovaSeq® 6000 (**v1.5** reagent kits), NextSeq® 1000, 2000 (using **v1** Sample Sheet), NextSeq® 500, 550, HiSeq® 3000, 4000, X, MiniSeq® (standard reagents), iSeq® 100

If pooling samples prepared using this protocol together with other types of samples that were prepared using some other library preparation workflow, please ensure that sample indexes are **unique** amongst all samples aimed to be sequenced together to avoid sample index collisions.

Quantification and pooling of NGS library

Since each sample has been barcoded with a unique sample index combination during the PCR step, they can be pooled and sequenced together on the same Illumina flow cell. There are some considerations for how to pool each sample that will affect how the sequencing read amounts are distributed between each sample.

Each sample contains a number of unique molecules generated throughout the workflow (originating from the AOCs bound to each cell), and amplified during PCR. Depending on the nature of the sample (cell type, degree of stimulation / activation etc), the number of unique molecules can vary between samples.

There are two strategies for pooling samples that can affect how the sequencing reads are allocated between each sample:

- 1. Equimolar pooling (recommended):** An equal number of moles are pooled together for each sample, resulting in an equal number of sequenced reads for each sample, regardless of the number of unique molecules present in each sample. Each sample is quantified and diluted to the same concentration separately, before pooled together.
- 2. Equivolume pooling:** An equal volume of purified PCR product is pooled together for each sample. The pool is then quantified and diluted for sequencing. This strategy will result in an equal number of reads per unique molecule, regardless of the sample, which means that the number of sequencing reads per sample will be proportional to the number of unique molecules.

Equivolume pooling strategy (2) can result in insufficient read depth for some samples if there is a high variability in the number of unique molecules between the samples. It is therefore recommended to perform equimolar pooling (1) which ensures equal sequencing read distribution per sample.

3. Workflow overview

The protocol described, shows how to perform the full workflow from live cells in suspension to a sequencing-ready NGS library. This protocol has been demonstrated and validated using peripheral blood mononuclear cells (PBMC), Raji and Jurkat cell lines.

Workflow illustration

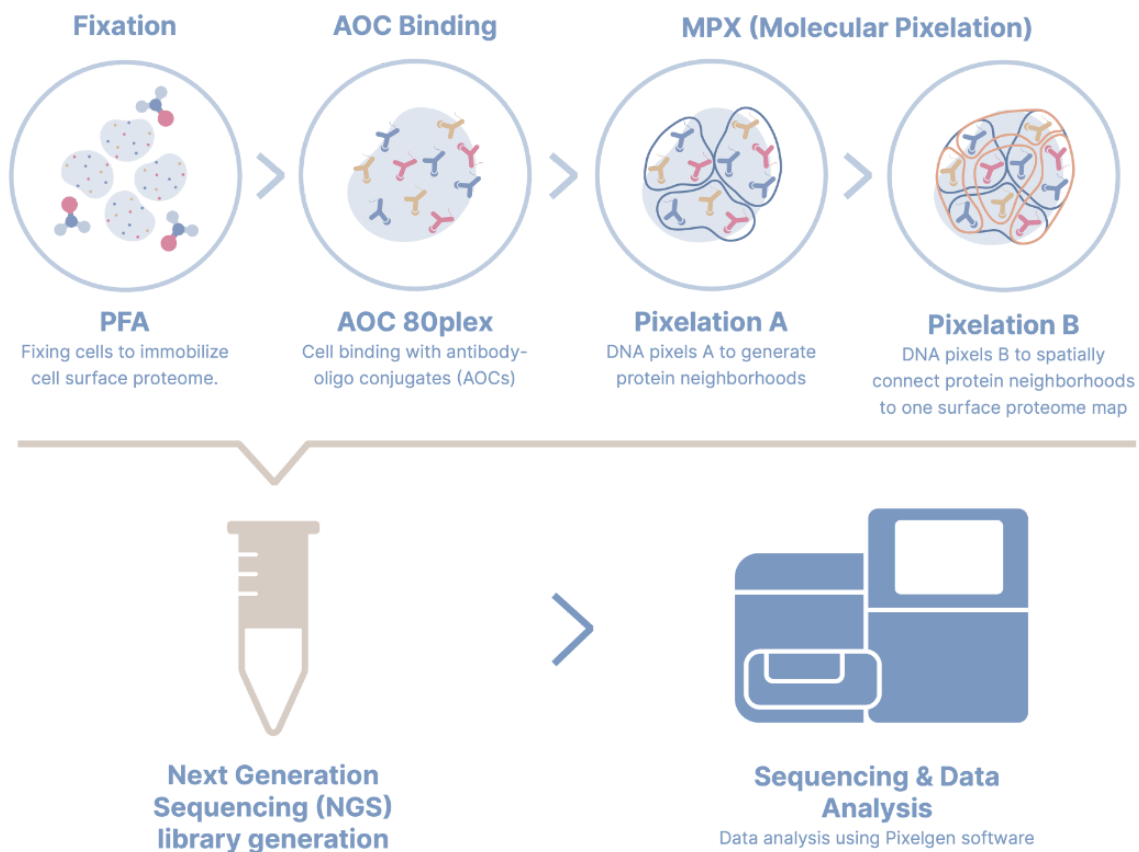






Figure 2. Overview of Molecular pixelation workflow.

There are 4 main steps in the protocol, starting from live cells in suspension to sequence-ready NGS library.

Table 5. Describes the workflow and outline each step and the approximate time needed.

Table 5. Workflow steps and time needed.

Step	Description	Duration
1	Cell preparation: <ul style="list-style-type: none"> • Cell fixation using PFA • Cell blocking and PFA quenching using Blocking Buffer  – Alt. stopping point: Up to 5 days at 4°C or up to 3 months at -80°C if performing the optional freezing protocol	1-2 h
2	Antibody-oligo conjugate (AOC) binding: <ul style="list-style-type: none"> • Binding of Antibody-oligo conjugates (AOCs) • Stabilization of bound AOCs using 2nd antibody • Cell counting and dilution  – Alt. stopping point: Up to 2 days at 4°C	3 h
3.1	Molecular pixelation (Pixelation A and B): <ul style="list-style-type: none"> • Hybridization of pixel set A • Gap-fill ligation A • Enzymatic removal of pixel set A – Alt. stopping point: Up to 1 h at 4°C <ul style="list-style-type: none"> • Hybridization of pixel set B • Gap-fill ligation step B  – Alt. stopping point: Up to 16 hours at 4°C	4-5 h
3.2	Molecular pixelation (Exonuclease and PCR): <ul style="list-style-type: none"> • Cell counting using hemocytometer and dilution • Exonuclease degradation of incomplete amplicons • PCR amplification and sample indexing  – Alt. stopping point: -20°C for long periods of time	3-4 h
4	NGS preparation: <ul style="list-style-type: none"> • PCR product cleanup using AMPure beads • Quantification and QC of NGS library 	1-2 h

4. Tips & Best Practices

General good lab practice

- Label tubes before starting protocol.
- Ensure centrifuge is balanced prior to loading samples.
- Use calibrated pipettes.
- It is recommended to process a maximum of 8 reactions in parallel.
- All incubations higher or equal to 37°C should have a heated lid temperature of 105°C.
- Aspirate at least 50% of the total reagent volume when pipette mixing to ensure proper mixing.
- A multichannel pipette can be used for wash buffer addition prior to centrifugation during wash steps. If doing so, ensure equal volumes are dispensed to each tube. To remove the liquid after centrifugation during wash steps, a single pipette needs to be used to not disturb the cell pellet.
- Make sure to never aspirate close to the bottom of the tube during liquid removal in wash steps - pellet will not be visible when working with low cell numbers.

General reagent handling

- Pulse-spin all reagent tubes before opening to pull down any liquid that may be present under the lid. Thoroughly **mix reagents before use** by pipetting up and down 10 times.
- Do not vortex samples or reagents.
- Keep all reagents on ice during setup and use, unless otherwise stated.
- Return enzymes, antibodies and buffers to their storage directly after use to minimize time exposed to elevated temperatures.
- Use the necessary precautions when handling paraformaldehyde (PFA) as it is a CMR substance (Carcinogenic, Mutagenic, or toxic to Reproduction).

5. Protocol

STEP 1 - Cell preparation

During Step 1 of the workflow, live cell suspensions are fixated using paraformaldehyde (PFA) and blocked using the Blocking solution.

Cell preparation

Note Check visually for cell aggregates or debris as these can contribute to inaccurate cell counting. If needed, filter the cell suspension using a cell strainer to remove large aggregates.

Note It is important to pipette the cell suspension gently throughout this part of the protocol.

- A. Count the cells using either automated cell counter (e.g., Countess II Automated Cell Counter), hemocytometer or other cell counting device, aiming at >90% viability.
- B. For each sample, transfer 500 000 - 1 million cells in <150 µl 1x PBS to separate PCR tubes.
- C. Centrifuge at 400 rcf for 4 min at 4°C.
- D. Carefully discard supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- E. Add 125 µl of 1x PBS on top of the 25 µl cell suspension, gently pipette up and down 10 times.
- F. Centrifuge at 400 rcf for 4 min at 4°C.
- G. Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- H. Add 65 µl of 1x PBS on top of the 25 µl cell suspension, gently pipette up and down 10 times or until the cells are uniformly suspended and proceed to Cell fixation and blocking.

Cell fixation and blocking

Note Use the necessary precautions when handling PFA solution since it is a CMR substance (Carcinogenic, Mutagenic, or toxic to Reproduction).

Note Prepare a fresh solution of 2% v/v PFA solution in 1xPBS. A volume of 100 μ l is needed per sample (90 μ l + extra). Use the solution within 2 hours, and store in dark until use.

- A. Add 90 μ l of the 2% PFA solution to each sample and pipette up and down 10 times or until the cells are uniformly suspended.
- B. Incubate for 15 min at room temperature (RT).
- C. Centrifuge at 700 rcf for 4 min at RT.
- D. From each 180 μ l fixation sample, carefully discard 155 μ l supernatant without disturbing the cell pellet, leaving behind 25 μ l of supernatant.
- E. Invert the **Wash Buffer** tube 10 times to mix. Add 125 μ l of **Wash Buffer** on top of the 25 μ l cell suspension and pipette up and down 10 times.
- F. Centrifuge at 700 rcf for 4 min at RT.
- G. Carefully discard 135 μ l supernatant without disturbing the cell pellet, leaving behind 15 μ l of supernatant.
- H. Add 165 μ l **Blocking Buffer** and resuspend the pellet by pipetting up and down 10 times.
- I. Incubate for 15 min at 4°C.
- J. Centrifuge at 700 rcf for 4 min at RT.
- K. Carefully discard 155 μ l supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.
- L. Add 125 μ l of **Wash Buffer** on top of the 25 μ l cell suspension and pipette up and down 10 times.
- M. Centrifuge at 700 rcf for 4 min at RT.
- N. Carefully discard 125 μ l supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.

- O. Add 125 μ l of 1x PBS on top of the 25 μ l cell suspension.
- P. Optional: Determine the cell concentration after PFA fixation, using either hemocytometer or other cell counting device (e.g. Countess II Automated Cell Counter).



Note At this step, cells can be stored at 4°C in 1xPBS for up to 5 days or frozen at -80°C (see optional below) until proceeding with AOC binding.

OPTIONAL: Freezing and thawing of fixed cells

This is an optional step, if planning to do AOC binding within 5 days, please proceed to *STEP 2 - Antibody-Oligo Conjugate (AOC) binding*.

The fixed and blocked cells can be frozen in -80°C for longer storage, up to 3 months. This part of the protocol describes the freezing and thawing procedure.

Freezing of PFA-fixed cells

Note Prepare a fresh freezing solution of 5% DMSO and 95% FBS. A volume of 600 μ l per sample is needed (500 μ l + extra).

- A. Centrifuge at 700 rcf for 4 min at RT and remove 125 μ l supernatant without disturbing the cell pellet, leaving behind 25 μ l.
- B. Add 125 μ l freezing solution and pipette up and down 10 times.
- C. Transfer the 150 μ l cell solution to a cryotube.
- D. Add 350 μ l freezing solution to the 150 μ l cell solution.
- E. Place the cryotubes in a cryogenic box and transfer to -80°C until further use.

Note At this step, cells can be kept in -80°C storage for 3 months.

Thawing of frozen cells

- F. Put the cryotubes in a 37°C bath for up to 5 minutes (confirm that the cell solution has thawed).
- G. Transfer the thawed 500 µl cell solution to a 1.5 ml Eppendorf tube.
- H. Add 500 µl **Wash buffer** to the empty cryotube and pipette up and down 10 times to wash any remaining cells.
- I. Transfer the 500 µl wash to the Eppendorf tube. Total of 1000 µl in each tube.
- J. Centrifuge at 700 rcf for 4 min at RT.
- K. Remove 950 µl supernatant without disturbing the cell pellet, leaving behind 50 µl.
- L. Add 950 µl **Wash buffer** and pipette up and down 10 times.
- M. Centrifuge at 700 rcf for 4 min at RT.
- N. Remove 950 µl supernatant without disturbing the cell pellet, leaving behind 50 µl.
- O. Add 100 µl 1x PBS to resuspend the pellet.
- P. Optional: Determine the cell concentration after thawing, using either hemocytometer or other cell counting device (e.g. Countess II Automated Cell Counter).



POSSIBLE
Up to 24 hours
at 4°C

Note At this step, cells can be kept at +4°C storage for 24 hours.

STEP 2 - Antibody-Oligo Conjugate (AOC) binding

Step 2 contains binding of Antibody-oligo conjugates (AOCs) to protein targets, and their stabilization on cells using a secondary antibody. Cells are counted and diluted at the end of this part of the workflow prior to starting the Molecular Pixelation steps.

Note From this step and forward, make sure cells become resuspended during pipette mixing steps by mixing vigorously. Too gentle pipette-mixing may result in cell aggregate formation.

AOC binding

- A. Centrifuge the vial containing **AOC panel** solution at 17000 rcf for 10 min at RT or 4°C, to pull down any aggregates to the bottom of the **AOC panel** vial.
- B. While centrifuging the AOC panel, centrifuge the cell suspensions in another centrifuge at 700 rcf for 4 min at RT.
- C. Carefully discard about 125 µl (less if cells were used for counting) sample supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- D. Add 25 µl of the centrifuged **AOC panel** solution to each 25 µl sample, to a final volume of 50 µl, and pipette up and down 10 times. Make sure to avoid aspirating near the bottom of the centrifuged **AOC panel** vial.
- E. Incubate for 45 min at 4°C.
- F. Add 100 µl **Wash Buffer** on top of the 50 µl cell suspension and pipette up and down 10 times.
- G. Centrifuge at 700 rcf for 4 min at RT.
- H. Carefully discard 125 µl supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.
- I. Add 125 µl **Wash Buffer** on top of the 25 µl cell suspension and pipette up and down 10 times.
- J. Centrifuge at 700 rcf for 4 min at RT.
- K. Carefully discard 125 µl of supernatant without disturbing the cell pellet, leaving behind 25 µl supernatant to preserve the pellet.

- L. Repeat steps I-K 2 more times, for a total of 4 washes (including F-H). It is important to perform in total 4 washes to completely remove any remaining unbound AOCs present in the solution.
- M. Quick spin the **2nd Antibody** and pipette it up and down 5 times to mix.
- N. Add 25 μ l of **2nd Antibody** to the 25 μ l cell pellet and pipette up and down 10 times.
- O. Incubate for 30 min at 37°C.
- P. Add 100 μ l **Wash Buffer** on top of the 50 μ l cell suspension and pipette up and down 10 times.
- Q. Centrifuge at 700 rcf for 4 min at RT.
- R. Carefully discard 125 μ l supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.
- S. Add 125 μ l **Wash Buffer** on top of the 25 μ l cell suspension and pipette up and down 10 times.
- T. Centrifuge at 700 rcf for 4 min at RT.
- U. Carefully discard 125 μ l supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.
- V. Resuspend the cell pellet in 75 μ l of 1xPBS and pipette up and down 10 times. Total of 100 μ l in each tube.
- W. Determine the cell concentration for each sample using either hemocytometer or other cell counting device (e.g Countess II Automated Cell Counter). Mix by pipetting up and down 10 times before taking an aliquot for counting.



POSSIBLE
Up to 2 days
at 4°C

Note Make sure to not use too big of a portion of the sample for counting, as that may result in a deficit of cell input to MPX (20 000 cells).

Note At this step, cells can be stored at 4°C in 1xPBS for up to 2 days before proceeding with the next steps of the protocol.

STEP 3.1 - Molecular Pixelation (Pixelation A and B)

In this step of the protocol, a series of DNA hybridization and enzymatic treatments are performed to incorporate DNA-pixel barcodes onto AOC oligos bound to the cells, forming the amplicons that can be amplified by PCR and finally sequenced. Reminder: quick spin all reagents and pipette each reagent tube up and down 5 times to mix before preparing the Master mix.

DNA-Pixel A hybridization

Note Recommended starting point is to load 20 000 cells per sample in 1xPBS to a total volume of 25 μ l.



Note The protocol can be paused for up to 1h between the steps Pixel A remover and DNA-Pixel B hybridization.

- A. Dilute cells in fresh PCR tubes to 800 cells/ μ l in 1x PBS for a total of 25 μ l (20 000 cells in total per sample).

Note If the cell concentration is lower than 800 cells/ μ l, centrifuge sample at 700 rcf for 4 min at RT and remove e.g. 50% of the volume to get 2x original concentration.

- B. Thaw the **Pixel A Diluent** and **Pixel A**, and prepare **Pixel A** hybridization master mix as indicated in Table 6. directly before use by combining the reagents in the order listed below, and maintain at RT.

Table 6. **Pixel A** hybridization master mix preparation.




Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
 Box2 -20°C	Pixel A Diluent	BB019A	16.5 μ l	66 μ l	132 μ l
 Box2 -20°C	Pixel A	PP018A	11 μ l	44 μ l	88 μ l
	Total		27.5 μ l	110 μ l	220 μ l

- C. Quick spin the **Pixel A** hybridization master mix and pipette up and down 5 times to mix.
- D. Dispense 25 μ l **Pixel A** hybridization master mix to each 25 μ l sample and pipette up and down 10 times. Total of 50 μ l in each tube.
- E. Incubate the samples in a thermal cycler for 15 min at 55°C.
- F. Remove the samples from the thermal cycler, add 100 μ l **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 μ l in each tube.
- G. Centrifuge at 700 rcf for 4 min at RT.
- H. Carefully discard 125 μ l of supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.
- I. To each sample, add 125 μ l of **Wash Buffer** and pipette up and down 10 times.
- J. Centrifuge at 700 rcf for 4 min at RT.
- K. Carefully discard 125 μ l of supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.

Gap-fill ligation step 1

- A. Thaw the **Gap-fill Buffer** and prepare **Gap-fill** master mix as indicated in Table 7. directly before use by combining the reagents in the order listed below and maintain at RT. Keep the **Gap-fill Buffer** on ice or at 4°C, until used at the second Gap-fill ligation step 2 of the protocol (put the enzymes back at -20°C until second Gap-fill ligation step).

Table 7. **Gap-fill** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
 Box2 -20°C	Gap-fill Buffer	BB018	25.3 μ l	101.2 μ l	202.4 μ l
 Box2 -20°C	Gap-fill Ligase	EE018	1.1 μ l	4.4 μ l	8.8 μ l
 Box2 -20°C	Gap-fill Polymerase	EE019	1.1 μ l	4.4 μ l	8.8 μ l
	Total		27.5 μ l	110 μ l	220 μ l

- B. Quick spin the **Gap-fill** hybridization master mix and pipette up and down 5 times to mix.
- C. Dispense 25 μ l **Gap-fill** hybridization master mix to each 25 μ l sample and pipette up and down 10 times. Total of 50 μ l in each tube.
- D. Incubate the samples in a thermal cycler for 20 min at 37°C.
- E. Add 100 μ l of **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 μ l in each tube.
- F. Centrifuge at 700 rcf for 4 min at RT.
- G. Carefully discard 125 μ l of supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.

Pixel A remover

- A. Prepare **Pixel A Remover** master mix according to Table 8. directly before use by adding the reagents in the order listed below and maintain at RT.

Table 8. **Pixel A Remover** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
Box1 +4°C	Wash Buffer	PP016	26.4 μ l	105.6 μ l	211.2 μ l
Box2 -20°C	Pixel A Remover	EE022	1.1 μ l	4.4 μ l	8.8 μ l
Total			27.5 μ l	110 μ l	220 μ l

- B. Quick spin the **Pixel A Remover** master mix and pipette up and down 5 times to mix.
- C. Dispense 25 μ l **Pixel A Remover** master mix to each 25 μ l sample and pipette up and down 10 times. Total of 50 μ l in each tube.
- D. Incubate the samples in a thermal cycler for 15 min at 37°C.

- E. Add 100 μl of **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 μl in each tube.
- F. Centrifuge at 700 rcf for 4 min at RT.
- G. Carefully discard 125 μl of supernatant without disturbing the cell pellet, leaving behind 25 μl supernatant to preserve the pellet.





POSSIBLE
Up to 1 hour
at 4°C

Note If pausing for up to 1 h at this step, resuspend the pellet by pipetting up and down 10 times before putting the samples at 4°C.

DNA-pixel B hybridization

- A. Thaw the **Pixel B Diluent** and **Pixel B**, and prepare **Pixel B** hybridization master mix as indicated in Table 9. shortly before use by adding the reagents in the order listed below and maintain at RT.

Table 9. **Pixel B** hybridization master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
 Box2 -20°C	Pixel B Diluent	BB019B	16.5 μl	66 μl	132 μl
 Box2 -20°C	Pixel B	PP015B	11 μl	44 μl	88 μl
	Total		27.5 μl	110 μl	220 μl




- B. Quick spin the **Pixel B** hybridization master mix and pipette up and down 5 times to mix.
- C. Dispense 25 μl **Pixel B** hybridization master mix to each 25 μl sample and pipette up and down 10 times. Total of 50 μl in each tube.
- D. Incubate the samples in a thermal cycler for 15 min at 55°C.
- E. Add 100 μl **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 μl in each tube.
- F. Centrifuge at 700 rcf for 4 min at RT.

- G. Carefully discard 125 μl of supernatant without disturbing the cell pellet, leaving behind 25 μl of supernatant to preserve the pellet.
- H. To each sample, add 125 μl **Wash Buffer** and pipette up and down 10 times.
- I. Centrifuge at 700 rcf for 4 min at RT.
- J. Carefully discard 125 μl of supernatant without disturbing the cell pellet, leaving behind 25 μl of supernatant to preserve the pellet.

Gap-fill ligation step 2

- A. Prepare **Gap-fill** master mix as indicated in Table 10. directly before use by adding the reagents in the order listed below and maintain at RT. Return potential remainders of the Gap-fill Buffer and enzymes to -20°C storage.

Table 10. **Gap-fill** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
 Box2 -20°C	Gap-fill Buffer	BB018	25.3 μl	101.2 μl	202.4 μl
 Box2 -20°C	Gap-fill Ligase	EE018	1.1 μl	4.4 μl	8.8 μl
 Box2 -20°C	Gap-fill Polymerase	EE019	1.1 μl	4.4 μl	8.8 μl
Total			27.5 μl	110 μl	220 μl

- B. Quick spin the **Gap-fill** master mix and pipette up and down 5 times to mix.
- C. Dispense 25 μl **Gap-fill** master mix to each 25 μl sample and pipette up and down 10 times. Total of 50 μl in each tube.
- D. Incubate the samples in a thermal cycler for 20 min at 37°C .
- E. Add 100 μl of **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 150 μl in each tube.

- F. Centrifuge at 700 rcf for 4 min at RT.
- G. Carefully discard 125 μ l of supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.
- H. To each sample, add 125 μ l of **Wash Buffer** and pipette up and down 10 times.
- I. Centrifuge at 700 rcf for 4 min at RT.
- J. Carefully discard 125 μ l of supernatant without disturbing the cell pellet, leaving behind 25 μ l supernatant to preserve the pellet.
- K. Add 50 μ l **Wash Buffer** to each sample at RT and pipette up and down 10 times. Total of 75 μ l in each tube.



POSSIBLE
Up to 16 hours
at 4°C

Note At this step, cells can be stored at 4°C for up to 16 hours before proceeding with the next steps of the protocol.

STEP 3.2 - Molecular Pixelation (Exonuclease & PCR)

Exonuclease treatment

- A. Count cells using a hemocytometer or single-use counting chamber. To ensure accuracy, count cells in replicates (2 reproducible counts). See *2. General Guidelines and Information: cell counting considerations* for more detailed information regarding cell counting.
- B. For each sample, transfer 300 to 1000 cells (depending on experimental set-up) in **Wash Buffer** to a final volume of 7 μ l, in new PCR tubes. See *Appendix 1* for an example table.

Note If the cell concentration is lower than 143 cells/ μ l, centrifuge sample at 700 rcf for 4 min at RT and remove e.g. 50% of the volume to get 2x original concentration.

- C. Prepare **Exonuclease** master mix as indicated in Table 11. directly before use by adding the reagents in the order listed below, pipette up and down 10 times and maintain at RT until use.

Table 11. **Exonuclease** master mix preparation.

Lid color, box #, box storage	Component	Article #	1x rxn + 10% extra	4x rxn + 10% extra	8x rxn + 10% extra
■ Box2 -20°C	Gap-fill Buffer	BB018	7.7 μ l	30.8 μ l	61.6 μ l
■ Box2 -20°C	Exonuclease	EE023	1.1 μ l	4.4 μ l	8.8 μ l
	Total		8.8 μ l	35.2 μ l	70.4 μ l

- D. Dispense 8 μ l **Exonuclease** master mix to each 7 μ l cell sample containing cells and pipette up and down 5 times to mix, total of 15 μ l in each tube.
- E. Incubate in a thermal cycler at 37°C for 30 min, followed by 75°C for 10 min (Table 12).

- F. Proceed to PCR.

Table 12. Incubation protocol for **Exonuclease** treatment.

Lid temperature	Reaction Volume	Run Time
105°C	15 µl	40 min
Step	Temperature	Duration
1	37°C	00:30:00
2	75°C	00:10:00
3	4°C	Hold

Sample index PCR

Sample indexes are introduced during PCR, allowing for pooling and sequencing multiple reactions in the same sequencing run. The reagent kit is supplied with 8 different sample indexes. Please see Paragraph 4 "General Guidelines & Information" in this document, for more detailed description and considerations related to sample indexing.

Note Make sure to use different index primers for each sample and note which sample index was used for each sample.

- A. Pre-program a PCR system with the PCR program denoted in Table 13.
- B. Thaw the **PCR Master Mix** vial (box 3) completely prior to use. Quick spin the **PCR Master Mix** and pipette up and down 5 times to mix.
- C. Add 5 µl of PCR index primer (box 3) to each 15 µl exonuclease treated sample.
- D. Add 20 µl of **PCR Master Mix** and pipette up and down 10 times to mix. Total of 40 µl per sample.
- E. Perform PCR using the following PCR program (Table 13.).

Table 13. PCR program.

Lid temperature	Reaction Volume	Run Time	
105°C	40 µl	~ 42 min	
Step	Temperature	Time	Number of cycles*
1	98°C	00:01:00	1
2	98°C	00:00:15	1000 cells: 13 cycles 500 cells: 14 cycles 300 cells: 15 cycles
3	60°C	00:00:30	
4	72°C	00:00:40	
5	72°C	00:05:00	1
6	4°C	Hold	

* Adjust number of PCR cycles with number of cells input to PCR reaction.



POSSIBLE
Up to 24 hours
at 4°C or long
term storage at
-20°C

Note The PCR products can be stored at 4°C for up to 24 hours or at -20°C for long term storage.

Note Quality control (QC) can be performed at this step, using an aliquot of the PCR product for gel electrophoresis. QC can also be performed after the AMPure beads clean-up, using instruments such as BioAnalyzer™, Tapestation™ or Fragment Analyzer™. See section *STEP 4 - Quantification and quality control of purified PCR products* for more information.

STEP 4 - NGS preparation

AMPure XP beads clean-up

The PCR products are purified using Ampure XP SPRI beads prior to NGS sequencing to remove primers, salts, dNTPs etc. Two consecutive rounds of AMPure cleanup is performed to ensure all primers have been removed, as any remaining primers will negatively affect sequencing results.

Third party Consumables and hardware required:

- AMPure XP (Beckman Coulter, cat. no. A63880)
- Invitrogen™ DynaMag™-96 Side Magnet (ThermoFisher, cat. no. 12331D)
- TE Buffer (10 mM Tris-Acetate pH 8.0, 1 mM EDTA) for DNA elution
- Freshly prepared 70% ethanol

Note Place Agencourt AMPure XP beads at RT for 30 min before use, as recommended by Beckman Coulter.

Note Always use freshly prepared 70% ethanol, as recommended by Beckman Coulter. Prepare 1 ml (720 µl + extra) 70% ethanol per sample.

Note It is important to perform two rounds of cleanup for complete removal of PCR primers.

- A. Vortex the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.
- B. For each sample, transfer 30 µl of PCR product to fresh PCR tubes. The remaining volume can be stored at -20°C as backup.
- C. Add 45 µl (1.5x bead:sample ratio) of resuspended Ampure bead mixture to each sample. Pipette mix 10 times to form a homogenous mixture.
- D. Incubate for 5 min at RT.

- E. Place the PCR tubes on a PCR tube magnet and incubate for 2 min, until the beads have settled against the tube wall.
- F. Carefully discard the supernatant, without disturbing beads.
- G. Add 180 μ l of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- H. Carefully remove the supernatant, without disturbing beads.
- I. Add 180 μ l of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- J. Carefully discard the supernatant, without disturbing beads.
- K. Completely remove the residual ethanol and air-dry beads with lid open until any remaining ethanol has evaporated.

Note Avoid over-drying the beads as this can negatively affect DNA recovery. The dried patch of beads will show some cracks if over-dried.

- L. Remove the PCR tubes from the magnet.
- M. Resuspend dried beads in 30 μ l of TE Buffer and incubate for 2 min at room temperature to elute the PCR product from the beads.
- N. Place the PCR tube on the magnet and incubate for 2 min, until the beads have settled against the tube wall.
- O. Carefully transfer the 30 μ l supernatant to new PCR tubes, without disturbing beads. Do not discard the supernatant in this step as it contains eluted product.
- P. Initiate cleanup round 2 by vortexing the Agencourt AMPure XP bottle and adding 45 μ l (1.5x bead:sample ratio) of resuspended Ampure bead mixture to each 30 μ l elution. Pipette mix 10 times to form a homogenous mixture.
- Q. Incubate for 5 min at RT.
- R. Place the PCR tubes on a PCR tube magnet and incubate for 2 min, until the beads have settled against the tube wall.
- S. Carefully discard the supernatant, without disturbing beads.
- T. Add 180 μ l of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- U. Carefully discard the supernatant, without disturbing beads.

- V. Add 180 μ l of 70% ethanol to the beads pellet while on the magnet and incubate for 30 sec.
- W. Carefully discard the supernatant, without disturbing beads.
- X. Completely remove the residual ethanol and air-dry beads with lid open until any remaining ethanol has evaporated.

Note Avoid over-drying the beads as this can negatively affect DNA recovery. The dried patch of beads will show some cracks if over-dried.

- Y. Remove the PCR tubes from the magnet.
- Z. Resuspend dried beads in 20 μ l of TE Buffer and incubate for 2 min at room temperature to elute the PCR product from the beads.
- AA. Place the PCR tube on the magnet and incubate for 2 min, until the beads have settled against the tube wall.
- BB. Carefully transfer the 20 μ l supernatant to new PCR tubes, without disturbing beads. Do not discard the supernatant in this step as it contains the purified PCR product.

The purified PCR products can either be prepared for sequencing directly, or stored at -20°C until use.

Quantification and quality control of purified PCR products

Before proceeding to NGS sequencing, samples need to be:

- Quality controlled (to confirm the correct size of approximately 268 bp)
- Quantified, diluted and pooled

Table 14. below describes what kits and instruments that can be used for this purpose. It is important to do both QC and quantification, i.e. if an instrument is used that only fulfills one of the purposes, another instrument needs to be included to perform the other.

Table 14. List of instruments that can be used for product quality control and/or quantification.

Instrument	Quality control	Quantification
BioAnalyzer™ with a High Sensitivity DNA kit (Agilent)	✓	✓
Fragment Analyzer™	✓	✓
TapeStation™	✓	✓
Qubit™ 4 Fluorometer with the Qubit™ dsDNA High Sensitivity (HS) Assay Kit (ThermoFischer)	✗	✓
Gel Electrophoresis with TBE gel, 200V for 25 min	✓	✗

The size of the generated PCR product is approximately 268 bp. To convert a concentration reading from ng/μl to nM, a conversion factor of 5.65 can be used:

$$\text{Library concentration [ng/}\mu\text{l]} \times 5.65 = X \text{ nM}$$

After QC and quantification, samples should be diluted and pooled. One method for performing **equimolar pooling** is to normalize each sample to the same concentration, followed by pooling by equal volumes of each normalized sample. The required concentration of the final pool for Illumina sequencing depends on the Illumina platform used. Creating a 2 nM pool will be sufficient for most Illumina platforms. For additional information, see Illumina guidelines for normalizing library concentrations [1].

The generated pool can be quantified again to ensure it is at the intended concentration.

The **loading concentration** for Illumina sequencing depends on the sequencing platform used. Illumina offers recommendations for loading concentrations for different library types.

- We recommend using the recommended loading concentration for “PhiX libraries”, as suggested by Illumina.

NGS sequencing using Illumina® platform

The sequenced amplicons contain regions of low diversity sequences, which can cause a quality drop on Illumina sequencing systems. Therefore each sequenced library should be spiked with:

15% PhiX to increase the diversity and base quality of low diversity regions. Please consult Illumina documentation for PhiX spiking.

Insufficient amount of sequencing reads per reaction will result in poor data quality.

Each reaction, corresponding to 300-1000 cells, should be given at least **120 000 reads/cell** to ensure good performance and to take into account cell counting variability.

Sequencing on NextSeq® 1000/2000 and NovaSeq® 6000 has been validated.

Table 15. denotes the recommended sequencing parameters. 28 cycles for read1 and 66 cycles for read2 are minimum values. Additional cycles may be used for read1 and read2 but will result in trimming of these additional bases during downstream Pixelator data processing.

Table 15. Sequencing parameters.

Sequencing depth	> 120 000 read pairs per cell
Sequencing type	Paired-end
Paired-end sequencing read configuration (minimum values)	Read1: 28 cycles Read2: 66 cycles i7 index: 8 cycles* i5 index: 8 cycles*

* Assuming sample indexes provided in reagent kit are used

Please see Pixelator tutorials [2] for description on data processing.

6. References

1. https://knowledge.illumina.com/library-preparation/general/library-preparation-general-reference_material-list/000001252
2. Pixelator data processing tutorials

Appendix 1

Dilution before Exonuclease treatment: example

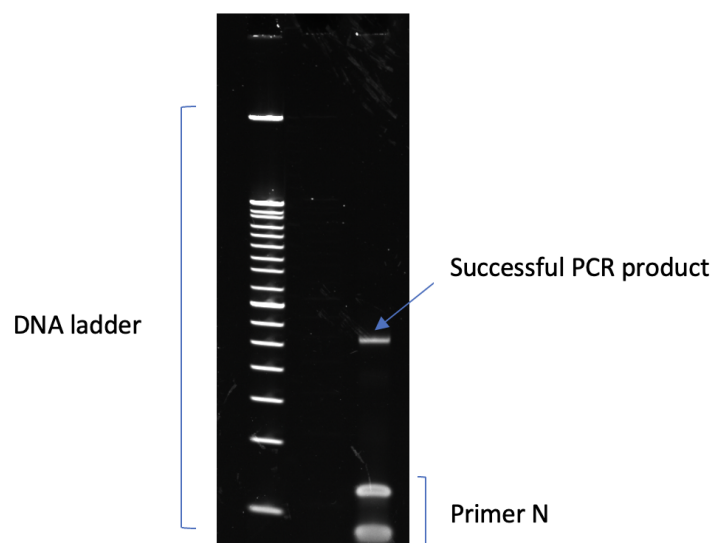
Appendix Table 1. displays an example of two samples with concentrations of 150 and 200 cells/ μ l and how to dilute them in Wash buffer to achieve 1000 cells per reaction in 7 μ l total.

Appendix Table 1. Example table of how to dilute cells before Exonuclease treatment, to achieve 1000 cells per reaction for sequencing.

Sample	Cell concentration	Sample volume needed	Wash buffer needed	Total
Sample 1	160 cells/ μ l	$1000/160 = 6.25 \mu$ l	$7-6.25 = 0.75 \mu$ l	$6.25+0.75 = 7 \mu$ l
Sample 2	200 cells/ μ l	$1000/200 = 5 \mu$ l	$7-5 = 2 \mu$ l	$5+2 = 7 \mu$ l

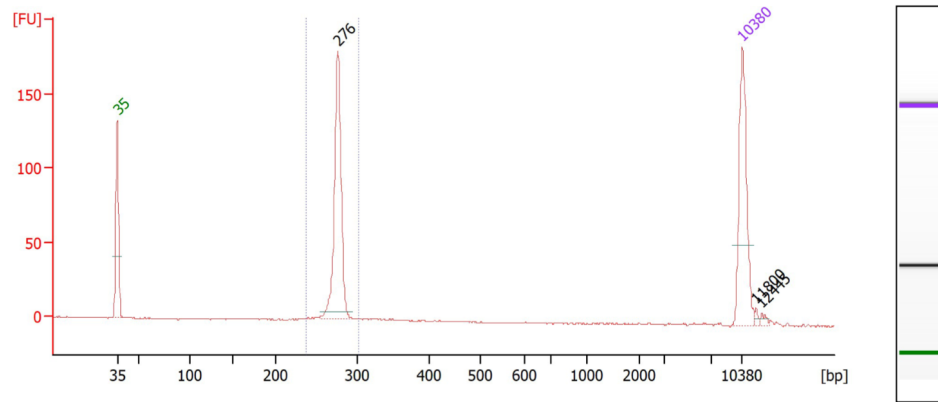
QC of NGS library size

The expected size of the generated PCR product is 268 bp. The presence of PCR products of the intended size can be confirmed using either gel electrophoresis or BioAnalyzer assay.



Appendix Figure 2. An example of a successfully amplified PCR product prior to AMPure cleanup using TBE gel electrophoresis. To the left - 50 bp

DNA ladder, to the right - 268 bp PCR product.



Appendix Figure 3. An example of a successfully amplified PCR product after AMPure cleanup using BioAnalyzer. The peak of the product is expected at around 268 bp.

Appendix 2

Protein Assay List

Product number: PXGIMM001

Summary: This list of 76 protein assays and 4 controls were chosen to deeply phenotype immune cells.

Number	Protein name	Gene name	UniProt #	CD antigen
1	Beta-2-microglobulin	β 2M	P61769	β 2M
2	Antigen-presenting glycoprotein CD1d	CD1D	P15813	CD1d
3	T-cell surface antigen CD2	CD2	P06729	CD2
4	T-cell surface glycoprotein CD3 epsilon chain	CD3E	P07766	CD3E
5	T-cell surface glycoprotein CD4	CD4	P01730	CD4
6	T-cell surface glycoprotein CD5	CD5	P06127	CD5
7	T-cell antigen CD7	CD7	P09564	CD7
8	T-cell surface glycoprotein CD8 a/b chains	CD8A/CD8B	P01732/P10966	CD8
9	CD9 antigen	CD9	P21926	CD9
10	Integrin α -L	ITGAL	P20701	CD11a
11	Integrin α -M	ITGAM	P11215	CD11b (ITGAM)
12	Integrin α -X	ITGAX	P20702	CD11c
13	Monocyte differentiation antigen CD14	CD14	P08571	CD14
14	Low affinity immunoglobulin gamma Fc region receptor III-A and -B	FCGR3A/FCGR3B	P08637/ O75015	CD16
15	Integrin beta 2	ITGB2	P05107	CD18
16	B-lymphocyte antigen CD19	CD19	P15391	CD19
17	B-lymphocyte antigen CD20	MS4A1	P11836	CD20
18	B-cell receptor CD22	CD22	P20273	CD22
19	Interleukin-2 receptor subunit alpha	IL2RA	P01589	CD25
20	Dipeptidyl peptidase 4	DPP4	P27487	CD26
21	CD27 antigen	CD27	P26842	CD27



Number	Protein name	Gene name	UniProt #	CD antigen
22	Integrin beta-1	ITGB1	P05556	CD29
23	Low affinity immunoglobulin gamma Fc region receptor II-b	FCGR2B	P31994	CD32
24	Myeloid cell surface antigen CD33	CD33	P20138	CD33
25	Complement receptor type 1	CR1	P17927	CD35
26	Platelet glycoprotein 4	CD36	P16671	CD36
27	Leukocyte antigen CD37	CD37	P11049	CD37
28	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1	CD38	P28907	CD38
29	Tumor necrosis factor receptor superfamily member 5	CD40	P25942	CD40
30	Integrin α -IIb	ITGA2B	P08514	CD41
31	Leukosialin	SPN	P16150	CD43
32	CD44 antigen	CD44	P16070	CD44
33	Receptor-type tyrosine-protein phosphatase C. All 6 isoforms (ABC, AB, BC, B and O)	PTPRC	P08575	CD45
34	Receptor-type tyrosine-protein phosphatase C. Isoform (ABC, AB and A)	CD45RA	P08575	CD45RA
35	Receptor-type tyrosine-protein phosphatase C. Isoform (ABC, AB, BC, and B)	CD45RB	P08575	CD45RB
36	Leukocyte surface antigen CD47	CD47	Q08722	CD47
37	CD48 antigen	CD48	P09326	CD48
38	Integrin α -4	ITGA4	P13612	CD49d
39	Intercellular adhesion molecule 3	ICAM3	P32942	CD50
40	CAMPATH-1 antigen	CD52	P31358	CD52
41	Leukocyte surface antigen CD53	CD53	P19397	CD53
42	Intercellular adhesion molecule 1	ICAM1	P05362	CD54
43	Complement decay-accelerating factor	CD55/DAF	P08174	CD55
44	CD59 glycoprotein	CD59	P13987	CD59
45	P-selectin	SELP	P16109	CD62P
46	High affinity immunoglobulin gamma Fc receptor I	FCGR1A	P12314	CD64



Number	Protein name	Gene name	UniProt #	CD antigen
47	Early activation antigen CD69	CD69	Q07108	CD69
48	Transferrin receptor protein 1	TFRC	P02786	CD71
49	B-cell differentiation antigen CD72	CD72	P21854	CD72
50	CD82 antigen	CD82	P27701	CD82
51	SLAM family member 5	CD84	Q9UIB8	CD84
52	T-lymphocyte activation antigen CD86	CD86	P42081	CD86
53	Intercellular adhesion molecule 2	ICAM2	P13598	CD102
54	Interleukin-7 receptor subunit alpha	IL7R	P16871	CD127 (IL-7R α)
55	Tumor necrosis factor receptor superfamily member 9	TNFRSF9 (CD137)	Q07011	CD137
56	Signaling lymphocytic activation molecule	SLAMF1	Q13291	CD150
57	Cytotoxic T-lymphocyte protein 4	CTLA4	P16410	CD152
58	CD40 ligand	CD40LG	P29965	CD154
59	Killer cell immunoglobulin-like receptor 2D	KIR2D	*)	CD158
60	Killer cell lectin-like receptor subfamily B member 1	KLRB1	Q12918	CD161
61	P-selectin glycoprotein ligand 1	SELPLG	Q14242	CD162
62	Scavenger receptor cysteine-rich type 1 protein M130	CD163	Q86VB7	CD163
63	C-C chemokine receptor type 7	CCR7	P32248	CD197
94	OX-2 membrane glycoprotein	CD200	P41217	CD200
65	T-lymphocyte surface antigen Ly-9	LY9	Q9HBG7	CD229
66	Natural killer cell receptor 2B4	CD244	Q9BZW8	CD244
67	Tumor necrosis factor receptor superfamily member 13C (BAFFR)	TNFRSF13C	Q96RJ3	CD268
68	Programmed cell death 1 ligand 1	CD274	Q9NZQ7	CD274 (PDL1)
69	Inducible T-cell co-stimulator	ICOS	Q9Y6W8	CD278
70	Programmed cell death protein 1	PDCD1	Q15116	CD279
71	NKG2-D type II integral membrane protein	KLRK1	P26718	CD314

Number	Protein name	Gene name	UniProt #	CD antigen
72	Natural cytotoxicity triggering receptor 3	NCR3	O14931	CD337
73	Sialic acid-binding Ig-like lectin 7 (SIGLEC-7)	SIGLEC7	Q9Y286	CD328
74	T-cell antigen receptor b (TCRB)		**)	
75	HLA class I histocompatibility antigen	HLA-ABC	O19689	HLA-ABC
76	HLA class II histocompatibility antigen, DR	HLA-DR	P01903/P01911	HLA-DR

*) This assay recognizes KIR2D members of the killer cell immunoglobulin (Ig)-like receptor (KIR) family, CD158a, CD158b and P50.3.

***) This assay recognizes the TCRB part of the T-cell receptor complex expressed by HPB-ALL cell line [carrying V(beta 5.3)] and a small subset of peripheral blood T cells.

CONTROL ASSAYS

Number	Protein name	Gene name	UniProt #	CD antigen
Intracellular controls				
1	β-actin	ACTB	P60709	-
Isotype controls				
1	Mouse IgG1, kappa Isotype Control (1 of 3)			
2	Mouse IgG2a, kappa Isotype Control (2 of 3)			
3	Mouse IgG2b, kappa Isotype Control (3 of 3)			

For more details visit www.pixelgen.com/technology

